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HSM-LUP-GYR

26 July 2004

European Patent Office
Gitschiner Str. 103
D-10969 Berlin
Germany
(Tel: +49 30 25901-840)

BY COURIER

Dear Sirs,

Re: LUPIN LTD
PCT International Application
No. PCT/IN 02/00192
Filed on: 20/09/2002

We respectfully refer to Written Opinion mailed on 21st June and our request for extension of time dated 12 July 2004 seeking time up to 21 September 2004.

A response to the Written Opinion is enclosed herewith. The response comprises:

1. A detailed reply regarding novelty and inventiveness of the present invention.
2. Clean copies of amended pages 4, 13 and claim 8
3. Comparison document

It is respectfully submitted that the detailed response submitted herewith clearly establishes novelty and inventiveness.

It is respectfully requested that the response is duly considered and a favourable International Preliminary Examination Report is established.

Yours truly,

H Subramaniam

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PCT/IN 02/00192

Applicants' file reference: HSM-LUP-GYR

European Patent Office
Gitschiner Str. 103
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Germany

PCT International Application **PCT/IN02/00192**	§ § §	HSM-LUP-GYR	No.
	§	Authorised Officer:	
Applicant: Lupin Limited	§ § §		

International Filing date: 20 September 2002
Priority date: 20 September 2002

Enclosed with this response are the following:

1. A detailed reply regarding novelty and inventiveness of the present invention.
2. Clean copies of amended pages 4, 13 and claim 8
3. Comparison document

The applicants' now specifically refer to the Written Opinion. The Written Opinion holds that the method Claim 6 lacks in novelty. It is therefore, believed that to the extent search has been conducted, all other claims are novel and inventive.

The Search Report cites several documents of which only D1, i.e., XP002242358 (U. H. Manjunatha *et al.*) is an **X** category citation. All other citations are only **A** category citations. Since these do not affect the novelty and inventiveness of the applicants' invention, only U. H. Manjunatha *et al.* is addressed herewith.

U. H. Manjunatha et.al., "Monoclonal antibodies to mycobacterial DNA gyrase A inhibit DNA supercoiling activity",*Eur. J. Bicochem.*, 200, 268, 2038-2046 (XP-00224358), (hereinafter referred to as D1)

The above-mentioned documents essential describes generation of several monoclonal antibodies (mABs), *viz.* MsGyrA:C3, MsGyrA: H11, and MsGYrA:E9 against the gyrase A subunit (Gyr A) of *Mycobacterium smegmatis* and the characetization of these monoclonal antibodies with respect to epitope recognition and interaction with the enzyme DNA gyrase.

It this connection, it is respectfully submitted that DNA gyrase is a type II topoisomerase (cellular enymes that function in the segregation of newly replicated choromosome pairs, chromosome condensation and in altering DNA superhelicity) exclusively found in prokaryotes, the only enzyme that can introduce negative supercoiling in a reaction that depends on ATP hydrolysis. The enzyme also catalyses a

number of other topological interconversions such as knotting-unknotting and catenation-decatenation.

Accordingly, D1 demonstrates inhibition/neutralization of *Mycobacterium smegmatis* DNA gyrase using the above-mentioned monoclonal antibodies as would be evident from Fig 7 and the description in lines 21-43, page 2043 and Fig 8 and the description in lines 1-4, left hand column of page 2044 of the document. There is, however, neither any demonstration nor any suggestion in the entire cited document that such an inhibition/neturalization of *Mycobgacterium smegmatis* enzyme utilizing the said monoclonal antibodies could be extrapolated to similar inhibiting/neutralization of *Mycobacterium tuberculosis* DNA gyrase.

Even though, the last paragraph in page 2045 of the document mentions the potential of the monoclonal antibodies and information may be contained in the document for development/designing of peptide inhibitors against DNA gyrase, ***such a development/design cannot be achieved through conventional mean eg., through procurement of reagents from vendors.*** A design of peptide inhibitors involves a careful and diligent strategy with elaborate methodology/procedure.

Against this background, the inventors of the subject PCT Application No. PCT/IN02/00192 have engineered the production of a single chain antibody, which exhibits useful properties for inhibiting DNA gyrase. This was achieved by employing hybridoma cell lines that secrete the monoclonal antibody MsGyrA:C3. The said single chain antibody, properties exhibited by it and the process for preparation thereof are novel, not hitherto reported, which form the inventive step of the present invention. Such an engineering of the monoclonal antibody, through a selective process outlined in Section E1 to E12 (pp 13-18) of the application and its properties/usefulness cannot be anticipated from the teachings embodied in the document, D1.

In other words, even if one can be expected to make (through engineering of the monoclonal antibody) the single chain antibody through the teachings of the document, D1, however, there is no guarantee that the single chain antibody thus produced or the peptide thereof would exhibit inhibitory properties towards. *M. tuberculosis DNA gyrase and therefore, it will not be obvious to a person skilled in the art to the read the teachings contained in D1 and practice the present invention.*

It is also respectfully submitted that in order to qualify as an X category citation, the teachings contained in the cited document should be equal to the novel features of the invention. In other words, a person skilled in the art should be able to practice the applicants' invention by reading the cited document. As mentioned above, the cited document does not teach the single chain antibody thus produced or the peptide thereof would exhibit inhibitory properties towards. *M. tuberculosis DNA gyrase.* Therefore, we respectfully request the learned Examiner to reconsider and withdraw the objection.

Further, the following amendment has been made to lines 15-20, page 13 of the specification should further clarify the applicants' position on the novelty and inventiveness of their Invention.

Lines 15-20, page 13 of the specification read as follows:

"E. cloning, sequencing and expression of a DNA sequence coding for neutralizing antibody gene and design of bioactive peptides.

This example describes the cloning and expression of a nucleic acid sequence coding for a DNA gyrase neutralizing monoclonal antibody, MsGyrA:C3. Based on the inhibition of gyrase by scFv:GyrA and utilizing sequence of the antibody, bioactive peptides were designed and their inhibition of mycobacterial DNA gyrase was tested".

The above paragraph has been corrected as follows (underlined).

"E. Cloning, sequencing and expression of a DNA sequence encoding for neutralizing antibody gene and design of bioactive peptides.

This example describes the cloning and expression of a nucleic acid sequence coding for a DNA gyrase neutralizing single chain antibody, scFvA:GyrA. Based on the inhibition of gyrase by scFv:GyrA and utilizing sequence of the antibody, bioactive peptides were designed and their inhibition of mycobacterial DNA gyrase was tested".

In adding, we bring to your attention the following mistakes in the specification, which need to be corrected/ amended at the appropriate time. These are;

a) Paragraph, Section E, lines 15-20 page 13 of the application, which reads as follows:

"E. cloning, sequencing and expression of a DNA sequence coding for neutralizing antibody gene and design of bioactive peptides.

This example describes the cloning and expression of a nucleic acid sequence coding for a DNA gyrase neutralizing single monoclonal antibody, MsGyrA:C3. Based on the inhibition of gyrase by scFv:GyrA and utilizing sequence of the antibody, bioactive peptides were designed and their inhibition of mycobacterial DNA gyrase was tested".

may be corrected as follows (underlined).

"E. cloning, sequencing and expression of a DNA sequence encoding for neutralizing antibody gene and design of bioactive peptides.

This example describes the cloning and expression of a nucleic acid sequence coding for a DNA gyrase neutralizing single chain antibody, scFvA:GyrA. Based on the inhibition of gyrase by scFv:GyrA and utilizing sequence of the antibody, bioactive peptides were designed and their inhibition of mycobacterial DNA gyrase was tested".

b) Line 19, Claim 6, of the specification, which reads as follows:

"... said monoclonal antibody, fusing said variable heavy chain region and light regions...."

has been corrected as follows (underlined).

"... said monoclonal antibody, fusing said variable heavy chain and light chain regions..."

c) Claim 8 of the specification, which reads as follows:

"A plasmid which inhibits the activity of DNA gyrase from M. smegmatis and M. tuberculosis ..... shown in seq. ID # 3 and 4 respectively"

has been corrected as follows (underlined).

"A plasmid characterised in that it encodes an engineered single chain antibody containing amino acid sequences for inhibiting the activity of DNA gyrase from M. smegmatis and M. tuberculosis, the said amino acid sequences being as shown in seq. ID # 3 and 4 respectively.

d) In page 4, line 18, the sentence" --- *supercoiling activity catalyzed by M. smegmatis and M. tuberculosis DNA gyrases ..."*

has been corrected as follows:

"supercoiling activity catalyzed by *M. tuberculosis* DNA gyrases by"

In view of the submissions presented above and the clarificatory amendments made to the specification and claims, it is respectfully submitted that claim 8 (as well as all other claims) are novel and inventive over the prior art.

Accordingly, we respectfully request that a favourable International Preliminary Examination Report be issued.

Yours faithfully,

H. Subramaniam

Enclosures
Corrected pages 4, 13
Corrected claims
Comparison pages showing amendments

In yet another further aspect, the present invention provides an amino acid sequence of the recombinant ScFV : GyraA protein as shown in Seq. ID # 2, which inhibits DNA gyrase from *M. smegmatis* and *M. tuberculosis*.

In yet another embodiment of the invention, said engineered single chain antibody contains an amino acid sequences which inhibit the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis,* said amino acid sequences having the Seq. ID # 3 and Seq. ID # 4 respectively.

In a further aspect, the present invention provides monoclonal antibodies, viz. MSGyrA:C3 and MSGyrA:H11, which inhibits DNA gyrase from fluoroquinolone resistant *M. smegmatis* and *M. tuberculosis*.

In yet another further aspect, the present invention provides hybridoma cell lines C3B3 and H11E1, which secrete the monoclonal antibodies, MSGyrA:C3 and MSGyrA:H11, which also inhibits DNA gyrase from *M. smegmatis* and *M. tuberculosis*.

The monoclonal antibody (mAb) described in this invention has been generated against GyrA subunit of *M. smegmatis* DNA gyrase. The mAb cross reacts with GyrA subunit from fast and slow growing mycobacteria (U. H. Manjunatha et. al., *Eur. J. Biochem.*, **2001**, 268, 2038-2046). The invention describes the inhibition of DNA supercoiling activity catalyzed by *M. tuberculosis* DNA gyrases by full-length mAb and its Fab and single chain antibody (scFv) fragments. The present invention also describes inhibition of DNA gyrase activity by peptides derived from scFv. The invention also deals with novel mechanism of DNA gyrase inhibition is distinct from that of other known DNA gyrase inhibitors.

DESCRIPTION OF THE FIGURES

Figure 1A : Specificity of interaction of mAb.

Figure 1B : Effect of mAbs on mycobacterial DNA gyrase supercoiling activity.

Figure 2A : Effect of MsGyrA:C3 on DNA binding.

Figure 2B : Effect of MsGyrA:C3 on DNA cleavage.

Figure 2C : Effect of MsGyrA:C3 on ATP hydrolysis.

Figure 2D : Effect of MsGyrA:C3 on ATP independent DNA relaxation reaction of mycobacterial DNA gyrase.

Figure 3A and 3B : Effect of MsGyrA:C3 on quinolone resistant M. *smegmatis* DNA gyrase.

inhibited at 3 μg/ml and 6 μg/ml concentrations of MsGyrA:C3 for quinolone sensitive (D^S) and quinolone resistant (D^R) enzymes respectively (Figure 3B). The twofold difference in the mAb concentration between D^S and D^R enzymes is attributed to reduced specific activity of D^R enzyme. DNA gyrase from ofloxacin resistant, highly virulent clinical isolate of *M. tuberculosis* (ICC-222) was also assayed for the effect of mAb. The purified enzyme has an IC_{50} of ~10 μg/ml for ciprofloxacin, where as the MsGyrA:C3 inhibited DNA gyrase supercoiling activity at 3.0 μg/ml, similar to that of *M. smegmatis* enzyme (Figure 3C). The absence of cross-resistance essentially emphasizes the mode of action of mAb to be distinct to that of quinolones. Similar to MsGyrA:C3, MsGyrA:H11 also inhibited ciprofloxacin resistant *M. smegmatis* DNA gyrase (Figure 3D). These data confirm the novel inhibition mechanism of gyrase by mAb. Absence of cross-resistance to fluoroquinolone resistant DNA gyrase by mAb, warrants the study of MsGyrA:C3 further as it could aid in countering the drug resistance problem.

E. Cloning, sequencing and expression of a DNA sequence encoding for neutralizing antibody gene and design of bioactive peptides

This example describes the cloning and expression of a nucleic acid sequence coding for a DNA gyrase neutralizing monoclonal antibody, scFv:GyrA.. Based on the inhibition of gyrase by scFv:GyrA and utilizing sequence of the antibody, bioactive peptides were designed and their inhibition of mycobacterial DNA gyrase was tested.

E1 ***: Cell culture and Isolation of RNA:***

Total RNA was isolated from the actively secreting mAb:C3 hybridoma cell line. Briefly, confluent hybridoma cells ($3X10^8$) were washed with ice cold IMDM medium and total RNA was extracted using TRIzol reagent (Life technologies Inc). RNA was purified using RNeasy QUIAGEN as per the manufacturer's protocol. The quality of RNA was confirmed by electrophoresis in a 1% formaldehyde agarose gel.

E2 ***: First-strand cDNA synthesis:***

The first-strand cDNA was synthesized from total RNA using the reverse transcription reaction (RT). For annealing, 5 μg of total RNA was incubated with 0.2 μg/ml of random hexamer oligonucleotide in a 10 μl reaction volume at 70°C for 5 minutes, followed by immediate chilling on ice. The annealed mix was incubated with 1 mM dNTP and 20 Units of Moloney Murine Leukemia Virus reverse transcriptase, (M-

Claims

1. An engineered single chain antibody, which inhibits the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*.
2. The engineered single chain antibody as claimed in claim 1 wherein it contains amino acid sequences for inhibiting the activity of DNA gyrase from *M. smegmatis and M. tuberculosis* said amino acid sequences having the Seq. ID # 3 and 4 respectively.
3. An engineered single chain antibody as claimed in Claim 1 wherein said antibody has a nucleotide sequence shown in Seq. ID # 1.
4. An engineered single chain antibody as claimed in Claim 1 wherein said antibody has an amino acid sequence shown in Seq. ID # 2.
5. A peptide having an amino acid sequence as shown in Seq. ID # 2.
6. A process for the preparation of an engineered single chain antibody which inhibits the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*, said process comprising preparing complimentary DNA (cDNA) from the corresponding hybridoma cell lines which secretes monoclonal antibody, amplifying from said cDNA, DNA fragments encoding variable heavy chain region and light regions of said monoclonal antibody, fusing said variable heavy chain and light chain regions of said DNA fragments, cloning said fused DNA fragment in a plasmid, transforming said plasmid into *E. Coli* host strain, inducing said transformed cells to express said engineered single chain antibody and purifying said engineered single chain antibody from the induced cell lysate.
7. Monoclonal antibodies, which inhibit DNA gyrase from fluoroquinolone resistant *M. smegmatis* and *M. tuberculosis*.
8. A plasmid characterised in that it encodes an engineered single chain antibody containing amino acid sequences for inhibiting the activity of DNA gyrase from *M. smegmatis and M. tuberculosis,* said amino acid sequences being as shown in Seq. ID # 3 and 4 respectively.

In yet another further aspect, the present invention provides an amino acid sequence of the recombinant ScFV : GyraA protein as shown in Seq. ID # 2, which inhibits DNA gyrase from *M. smegmatis* and *M. tuberculosis*.

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3. An engineered single chain antibody as claimed in Claim 1 wherein said antibody has a nucleotide sequence shown in Seq. ID # 1.
4. An engineered single chain antibody as claimed in Claim 1 wherein said antibody has an amino acid sequence shown in Seq. ID # 2.
5. A peptide having an amino acid sequence as shown in Seq. ID # 2.
6. A process for the preparation of an engineered single chain antibody which inhibits the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*, said process comprising preparing complimentary DNA (cDNA) from the corresponding hybridoma cell lines which secretes monoclonal antibody, amplifying from said cDNA, DNA fragments encoding variable heavy chain region and light regions of said monoclonal antibody, fusing said variable heavy chain ~~region~~ and light chain regions of said DNA fragments, cloning said fused DNA fragment in a plasmid, transforming said plasmid into *E. Coli* host strain, inducing said transformed cells to express said engineered single chain antibody and purifying said engineered single chain antibody from the induced cell lysate.
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